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Site-specific incorporation of photofunctional nonnatural amino acids into a polypeptide through in vitro protein biosynthesis

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Abstract

Nonnatural amino acids with photofunctional groups were incorporated site-specifically into a polypeptide by using in vitro protein synthesizing system. The nonnatural amino acids were attached to $tRNA_{CCU}$ through chemical misacylation method, and added to the in vitro system with a mRNA containing a single AGG codon. L-*p*-Phenylazophenylalanine, L-2-anthrylalanine, L-1-naphthylalanine, L-2-naphthylalanine and L-*p*-biphenylalanine were successfully incorporated into a polypeptide, but L-1-pyrenylalanine was not. The polypeptides containing the nonnatural amino acids showed photofunctionalities.

Key words: Nonnatural amino acid; Photofunction; Protein biosynthesis; Minor codon; Phenylazophenylalanine; Anthrylalanine

1. Introduction

Incorporation of functional amino acids is an important strategy for the creation of artificial proteins. In our previous studies, α-helical polypeptides containing a variety of nonnatural amino acids were chemically synthesized and their conformations, the efficiencies of photo-induced electron transfer, and the photoresponsible structural changes have been investigated [1]. These studies showed that incorporation of nonnatural residues into carefully designed amino acid sequences can create novel functional polypeptides. By introducing these amino acids into proteins, the possibility of protein engineering will be greatly expanded. However, chemical synthesis of large proteins is still very difficult, although a few successful attempts have been reported [2]. Therefore, a biosynthetic strategy will be the best choice for the preparation of large proteins containing nonnatural amino acids.

Nonnatural amino acids that have similar structures to one of the 20 amino acids are known to be incorporated into proteins through misrecognition by aminoacyl tRNA synthetase. For example, norleucine has been successfully incorporated into human epidermal growth factor instead of methionine [3]. However, this technique may be applicable only to limited types of nonnatural amino acids and is not site specific. Site-specific incorporation of a wide variety of nonnatural amino acids can be accomplished by the use of chemically misacylated tRNAs [4]. This method has been applied to a model polypeptide [5], β-lactamase [6], T4 lysozyme [7], etc. However, nonnatural amino acids that are successfully incorporated so far, are limited to those carrying relatively small residues, except for a few cases [8]. One of the reasons may be that such amino acids are not accepted by the ribosomal system. We have examined the adaptability of various aromatic nonnatural amino acids to *E. coli* ribosomal A site [9], and found that nonnatural amino acids such as L-*p*-phenylazophenylalanine and L-2-anthrylalanine were good substrates of the ribosomal system.

Another problem in the previous system is that no more than two types of amino acids can be incorporated, since there are only three stop codons in the genetic code. The expansion of the genetic code has been attempted [10], but the replication and transcription step are still restrictive. The use of sense codons will resolve this problem. In the *E. coli* biosynthetic system, very few amount of $tRNA_{CCU}$ must be present, since the codon AGG appears scarcely in the system (less than 3%) [11]. Therefore, a chemically misacylated $tRNA_{CCU}$ will read the minor codon instead of endogenous arginyl $tRNA_{CCU}$.

In this report, we show that several nonnatural amino acids with photofunctional groups are incorporated into a polypeptide through *E. coli* in vitro protein synthesis system by the use of the chemically misacylated

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$tRNA_{CCU}$. Some photofunctions of the resulting polypeptides are also described.

2. Materials and methods

L-1-Naphthylalanine, L-2-naphthylalanine were purchased from Sigma. Other nonnatural amino acids, i.e. L-*p*-phenylazophenylalanine, L-1-pyrenylalanine, L-2-anthrylalanine and L-*p*-biphenylalanine were synthesized in our laboratory [1].

pdCpA's aminoacylated at the 3′ position with different nonnatural amino acids were synthesized as described before [12]. The aminoacyl dinucleotide was linked to a $tRNA_{CCU}$(-CA) that lacks the pCpA unit at the 3′ terminal, to prepare aminoacyl $tRNA_{CCU}$ of full length. The $tRNA_{CCU}$(-CA) was prepared from the corresponding DNA through run-off transcription.with T7 RNA polymerase [13]. The base sequence of the tRNA was taken from $tRNA^{Ile}$ except that the anticodon region is CCU. Also the identity determinants of the tRNA against arginyl tRNA synthetase (A_{20} and A_{74}) [14] were replaced by U_{20} and C_{74}, to inhibit the aminoacylation with arginine. The total sequence of the $tRNA_{CCU}$ is: G-G-G-C-U-U-G-U-A-G-C-U-C-A-G-G-U-G-G-U-U-A-G-A-G-C-G-C-A-C-C-C-C-U-C-C-U-A-A-G-G-G-U-G-A-G-G-U-C-G-G-U-G-G-U-U-C-A-A-G-U-C-C-A-C-U-C-A-A-G-C-C-C-C-C-(dC-A-amino acid). The $tRNA_{CCU}$(-CA) was coupled with various aminoacyl pdCpA by T4 RNA ligase. The detail of the design and synthesis of the tRNA will be reported elsewhere. [15]

mRNA was designed to contain single AGG and no other codon of arginine. The mRNA was transcribed from a synthetic gene [16] by T7 RNA polymerase. The sequence of the coding region is ATG-GCT-GGC-ATG-ACT-GGT-GGA-CAG-CAA-ATG-GGT-ACC-GTT-GTT-AGG-GTT-GTT-AAA-GCT-TAT-GTT-GTA-GTT-GTA-GTT-GTG-GTT-GTA-GTT-GTC-GTT-GTG-GTA-GCT-TAT-GTT-GTA-GTT-GTA-GTT-GTG-GTT-GTA-GTT-GTC-GTT-GTC-GTA-GCT-TAA-TAG-TGA. The sequence contains the AGG codon at the 15th position and 2 and 28 codons for valine before and after the 15th position, respectively.

The protein synthesis was carried out by using *E. coli* S-30 system [17]. The reaction mixture contained 55 mM HEPES-KOH, pH 7.5, 210 mM potassium glutamate, 6.9 mM ammonium acetate, 7.7 mM magnesium acetate, 1.7 mM dithiothreitol, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9% polyethyleneglycol-8000, 35 μg/ml folinic acid, 0.36 mM each of amino acids except valine and arginine, 18 μM [^{14}C]valine(290 mCi/mmol), 0.16 μg/ml *E. coli* tRNA, 400 μg/ml mRNA, 200 μl/ml *E. coli* S-30 extract and 25 μM aminoacyl $tRNA_{CCU}$. In the competition experiment, 18 μM [^{14}C]valine (29 mCi/mmol) and 1.9 μM [^{3}H]arginine (54 Ci/mmol) were added instead of [^{14}C]valine (290 mCi/mmol). 10 μl of the mixture was incubated at 37°C for 30 min, then the amount of polypeptide synthesized was evaluated by paper disk method [18].

In the case of L-2-anthrylalanine, 30 μl of reaction mixture was alkali-treated for deacylation of anthrylalanyl $tRNA_{CCU}$. After neutralization, the mixture was desalted with Bio-Gel P-100 equilibrated with phosphate buffer for exclusion of free anthrylalanine. The polypeptide was eluted at the void volume, since it formed high molecular weight aggregate in aqueous solution. Desalted crude solution was diluted to 1 ml, then fluorescence spectrum was measured at λ_{ex} = 255 nm. The exclusion of free anthrylalanine was confirmed by a control experiment that included same concentration of free anthrylalanine in the mixture.

In the case of L-*p*-phenylazophenylalanine, 1 μl of the reaction mixture treated with alkali was spotted onto nitrocellulose membrane, then washed with TBS (20 mM Tris-HCl, pH 7.5, 200 mM NaCl) containing 20% methanol. After blocking by gelatin, the membrane was incubated with an antibody [19] solution at 37°C, followed by washing with TBS containing 0.05% Tween-20. Peroxidase-labeled anti-mouse IgG was used as a second antibody and spots were colored by 4-chloro-1-naphthol/H_2O_2.

3. Results and discussion

The amounts of the polypeptides synthesized in the presence of various misaminoacylated $tRNA_{CCU}$'s are compared in Fig. 1. Without adding aminoacyl tRNA or with the addition of nonacylated $tRNA_{CCU}$, the protein synthesis was incomplete. Addition of phenylalanyl $tRNA_{CCU}$ increased the yield of the polypeptide. Addition of $tRNA_{CCU}$ charged with a nonnatural amino acid, L-*p*-phenylazophenylalanine, also increased the yield. However, addition of $tRNA_{CCU}$ charged with L-1-pyrenylalanine was not effective. The incomplete synthesis in the absence of aminoacyl $tRNA_{CCU}$ would be understood as an incomplete translation of the AGG codon. Since the aminoacyl $tRNA_{CCU}$ can read this *missense* codon, a successful protein synthesis will be expected in the presence of properly aminoacylated $tRNA_{CCU}$. Thus, the results in Fig. 1 indicate that phenylazophenylalanine is incorporated into the polypeptide but pyrenylalanine is not.

To confirm that arginine was replaced by the nonnatural amino acid, a small amount of [^{3}H]arginine was added into the translation system. As shown in Fig. 2, the ^{3}H content in the polypeptide decreased with the increase of the amount of phenylazophenylalanyl $tRNA_{CCU}$, whereas ^{14}C content, representing total amount of polypeptide, was constant. Since there is no other codon for arginine in the mRNA, this result strongly suggests that the chemically acylated $tRNA_{CCU}$ reads AGG codon in place of arginyl $tRNA_{CCU}$.

The content of [^{3}H]arginine in the presence of an excess amount of phenylazophenylalanyl $tRNA_{CCU}$ is still large. Therefore, the rather high ^{14}C contents in the top two lanes in Fig. 1 may be interpreted in terms of rather high yield of the polypeptide containing arginine at the

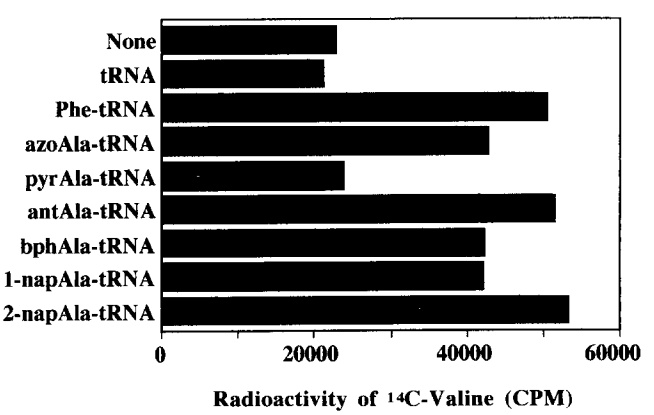

Fig. 1. Relative yields of polypeptides synthesized in the presence of chemically misaminoacylated $tRNA_{CCU}$ carrying different nonnatural amino acids. The polypeptides were translated in vitro as described in the text and the radioactivity of [^{14}C]valine contained in the acid precipitated fracton was counted as a measure of the product yield. None: no tRNA was added, tRNA: nonacylated $tRNA_{CCU}$ was added, Other Xyz-tRNA: $tRNA_{CCU}$ charged with L-phenylalanine (Phe), L-*p*-phenylazophenylalanine (azoAla), L-1-pyrenylalanine(pyrAla), L-2-anthrylalanine (antAla), L-*p*-biphenylalanine (bphAla), L-1-naphthylalanine (1-napAla), and L-2-naphthylalanine (2-napAla) were added, respectively.

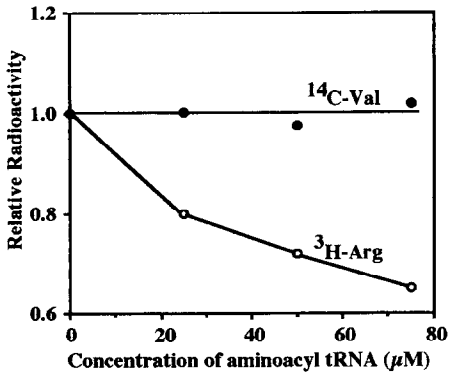

Fig. 2. Competition between phenylazophenylalanyl $tRNA_{CCU}$ and ^{3}H-labeled arginyl $tRNA^{Arg}_{CCU}$ for a translation of the AGG codon. As in the case of Fig. 1, the total yield of the polypeptide was evaluated from ^{14}C-labeled valine. The radioactivities found in the acid precipitated fraction were plotted against the concentration of L-*p*-phenylazophenylalanyl $tRNA_{CCU}$, after normalization by the values in the absence of the latter. The relative radioactivity of ^{3}H (○) corresponds to the fraction of arginine-containing polypeptides and that of ^{14}C (●) corresponds to the total yield of the polypeptide.

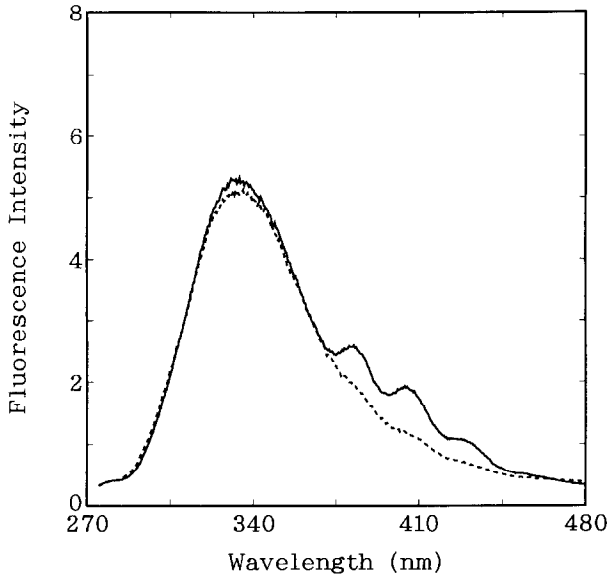

Fig. 3. Fluorescence spectra of the polypeptide synthesized in the presence of L-2-anthrylalaninyl $tRNA_{CCU}$ (——) and in the presence of free L-2-anthrylalanine (...) in phosphate buffer. In both cases, the solution containing the polypeptide was gel-filtrated to remove free anthrylalanine before measurement.

15th position. These facts indicate that the activity of minor $tRNA^{Arg}_{CCU}$ contained in the present in vitro biosynthetic system is still high. To improve the efficiency of substitution with nonnatural amino aicds, it will be necessary to suppress the activity of $tRNA^{Arg}_{CCU}$.

Other nonnatural amino acids were also examined. Addition of tRNA charged with L-2-anthrylalanine, L-*p*-biphenylalanine, L-1-naphthylalanine, or L-2-naphthylalanine increased the yield of the polypeptide, suggesting these amino acids were successfully incorporated into the polypeptide. As described above, however, L-1-pyrenylalanine could not be incorporated.

The selectivity of the nonnatural amino acids by the synthesizing system may be explained in terms of the adaptability to the active center of ribosomal A site as proposed in our previous report [9]. The nonnatural amino acids which were found to adapt to the ribosome were successfully incorporated in the present study. On the other hand, the incorporation of L-1-pyrenylalanine that could not adapt to the ribosome, was unsuccessful. L-1-Naphthylalanine could not adapt to the ribosome, but it was incorporated into polypeptide, although the efficiency was lower than that of 2-naphthylalanine. The case of L-1-naphthylalanine suggests that some amino acids that are not accepted by the ribosomal A site can be forced to the active site when they were charged on tRNA.

Incorporation of 2-anthrylalanine was directly confirmed by the fluorescence spectroscopy. Fluorescent spectrum (Fig. 3) of a crude polypeptide mixture obtained in the presence of anthrylalanyl $tRNA_{CCU}$ showed clear peaks characteristic of anthryl group, indicating the presence of anthrylalanine in the polypeptide. No anthryl fluorescence was observed in the polypeptide mixture obtained in the presence of free anthrylalanine instead of anthrylalanyl $tRNA_{CCU}$.

The incorporation of phenylazophenylalanine was confirmed by a immunochemical method using an antibody against an azobenzene group [19]. The antibody has been shown to bind to *trans* phenylazophenyalanine, and not to bind to the *cis* form. The synthesized polypeptide was spotted onto nitrocellulose membrane and detected by the antibody. As shown in Fig. 4, the binding of the antibody to the polypeptide was observed. Furthermore, irradiation of UV light to isomerize the azobenzene group to *cis* form before spotting the polypeptide, resulted in the decrease of the amount of antibody bound to the polypeptide. This indicates that the polypeptide forms a complex with the antibody under the control of light signal. The result suggests that a combination of azobenzene-containing proteins and anti-*trans*-azobenzene antibody provides a novel photoswitching ability in a variety of biological systems [20].

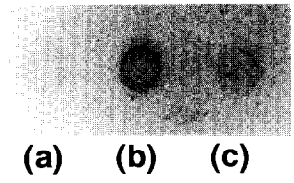

Fig. 4. Detection of *trans*-azobenzene group in the polypeptides synthesized in the absence (a) or in the presence (b) of L-*p*-phenylazophenylalanyl $tRNA_{CCU}$. Spot (c) is the same sample as (b) irradiated under UV light before spotting. The azobenzene group was detected on a nitrocellulose membrane with the use of an antibody against *trans*-L-*p*-phenylazophenylalanine.

In conclusion, several type of nonnatural amino acids carrying large photofunctional groups were incorporated into a polypeptide site-specifically corresponding to the AGG codon. This result will open a way to prepare proteins with novel photofunctionalities.

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